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(54) **METHOD FOR REDUCING CYANURIC ACID  
IN RECREATIONAL WATER SYSTEMS**

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31, 2014, provisional application No. 62/101,741,  
filed on Jan. 9, 2015.

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**C02F 3/34** (2006.01)  
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**C02F 1/70** (2006.01)  
**C02F 101/38** (2006.01)  
**C02F 103/42** (2006.01)

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CPC . **C02F 3/341** (2013.01); **C02F 1/70** (2013.01);  
**C02F 2101/38** (2013.01); **C02F 2103/42**  
(2013.01); **C02F 2305/06** (2013.01); **C12N 1/20**  
(2013.01)

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C02F 2305/06; C02F 2103/42; C12N 1/00;  
C12N 1/20; C12N 11/10  
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435/243, 252.31, 252.5, 262, 262.5  
See application file for complete search history.

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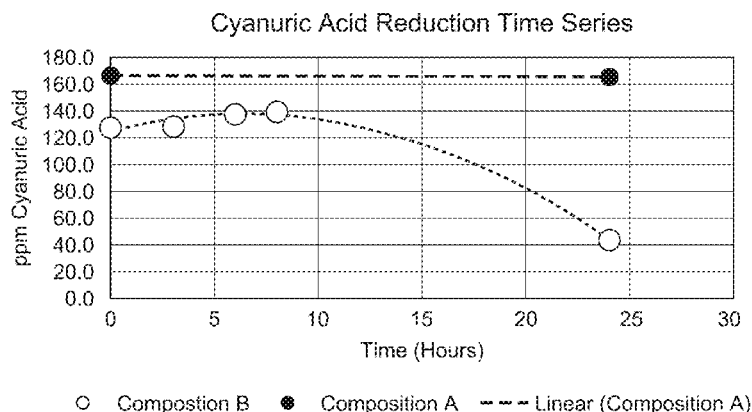
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(57) **ABSTRACT**

The present invention provides compositions and methods of  
reducing cyanuric acid levels in recreational water systems.

**15 Claims, 10 Drawing Sheets**



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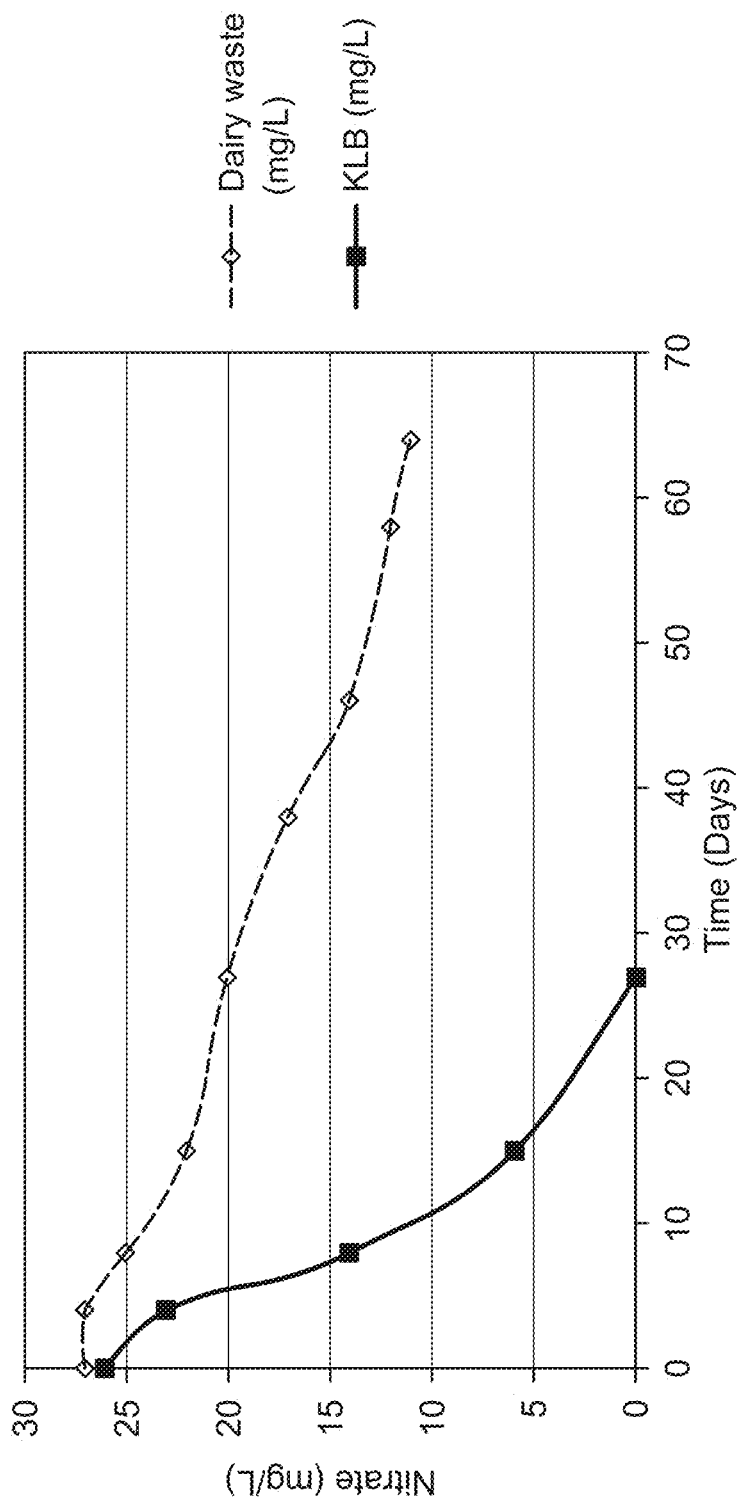
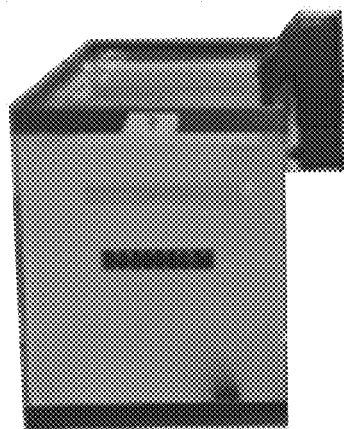
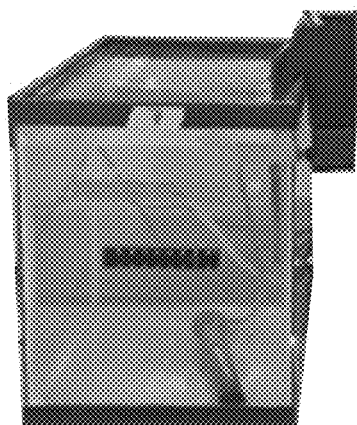


Fig. 1

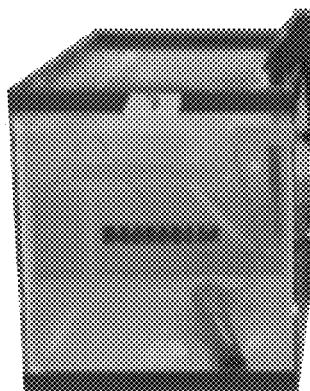
Fig. 2



Day 0



Day 1



Day 2

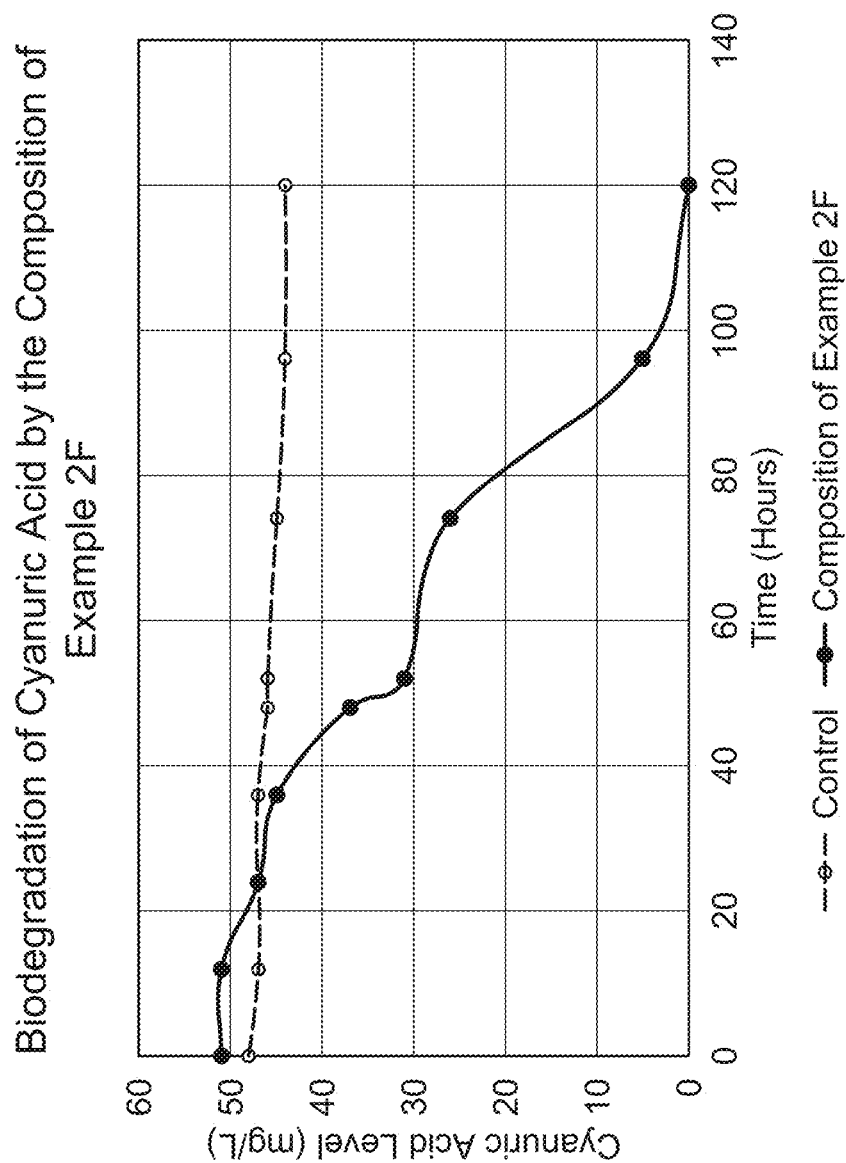


Fig. 3

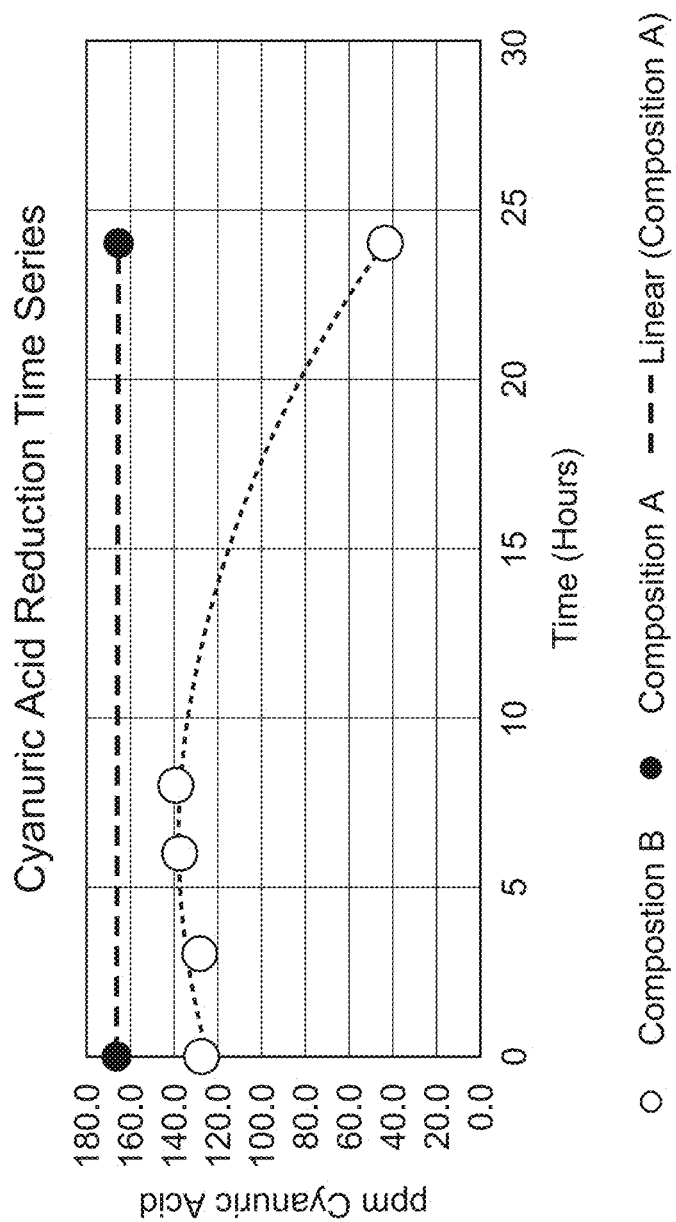
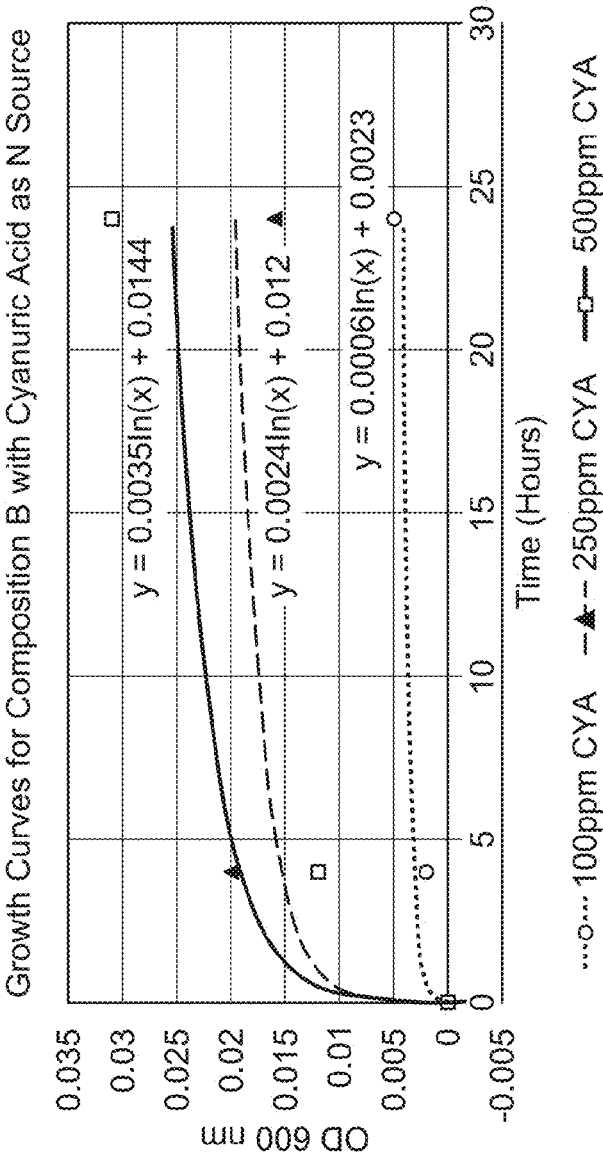


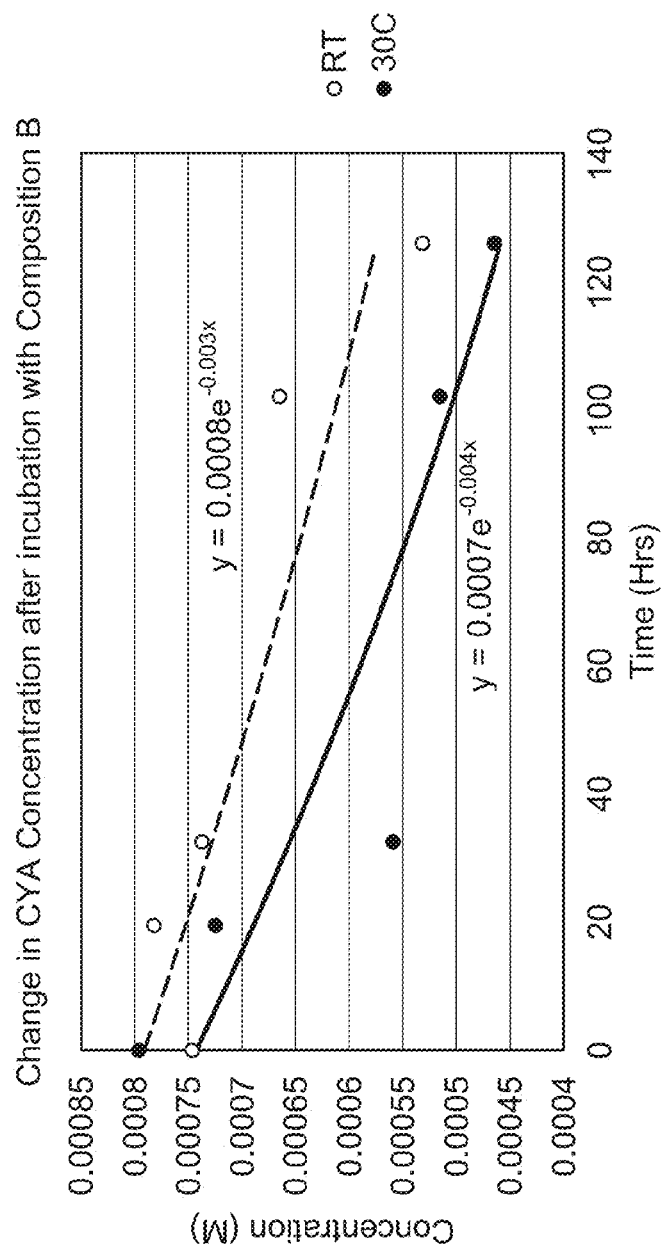
Fig. 4

Growth Curves for Composition B with Cyanuric Acid as Nitrogen Source



1. Composition B dosage = 10 ppm
2. Incubation at 40°C/150 rpm
3. Bacterial growth measured as OD at 600nm

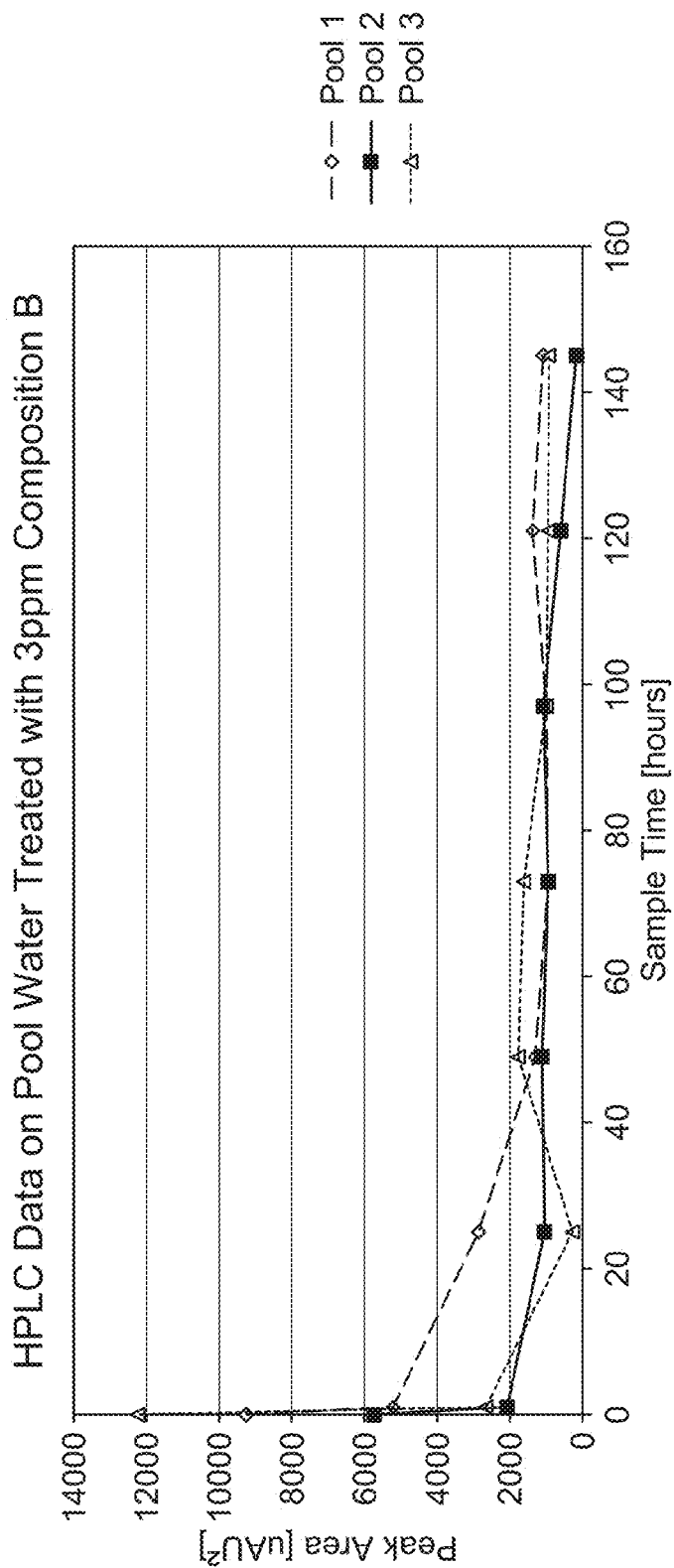
Fig. 5



1. Initial Cyanuric Acid Concentration = 100 ppm
2. Composition B dosed at 30 ppm
3. Cyanuric acid concentration measured via absorbance at 214 nm

Fig. 6

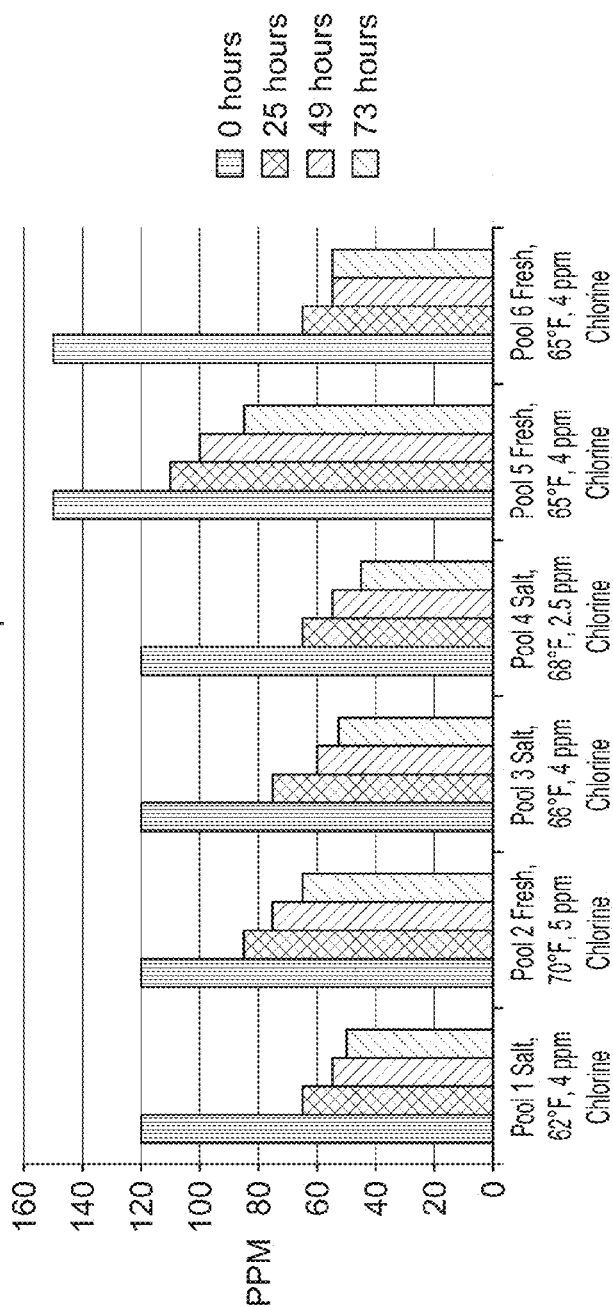




1. Initial Cyanuric Acid levels varied by pool but all pools started with CYA levels > 100ppm
2. Composition B dosed at 3 ppm

Fig. 7

# Reduction in Cyanuric Acid of Commercial Swimming Pools Treated with Composition B



Composition B was added to each pool at 3 ppm.

Cyanuric acid levels were measured every 24 hours out to 73 hours using CYA Test kit (precipitation assay).

Final Cyanuric Levels ranged between 45-85 ppm.

A 43%-63% Reduction. Note: In Pools 5 and 6, Cyanuric Acid

levels may be even higher than 150 ppm based on upper limits to the standard test method.

Fig. 8

Results of Florida Field Trials

	Pool #1	Pool #2	Pool #3
Temperature	60F	65F	55F
Dosage of Composition B (ppm)	2.2	3.0 ppm	3.0 ppm
Initial CYA Level (ppm)	180	>200	>200
CYA Level at 3 hrs (ppm)		100	150
CYA Level at 24 hrs (ppm)	100	90	90

Fig. 9

## Cyanuric Acid Reduction of Compositions A, B, and C Formulations

Pool #1	Pool #2	Pool #3
<u>Product Dosage</u>	<u>Product Dosage</u>	<u>Product Dosage</u>
4.3 ppm Composition B	3.7 ppm Composition A	3.7 ppm Composition C
Pool Capacity = 14,000 gal.	Pool Capacity = 16,000 gal.	Pool Capacity = 16,000 gal.
82°F	80°F	85°F
pH: 7.8	pH: 7.6	pH: 7.6
Initial CYA level = +150 ppm	Initial CYA level = 120 ppm	Initial CYA level = 120 ppm
CYA level at 25 hours = 55 ppm	CYA level at 25 hours = 65 ppm	CYA level at 25 hours = 75 ppm
<b>% CYA Reduction &gt;63%</b>	<b>% CYA Reduction &gt; 45%</b>	<b>% CYA Reduction &gt; 37%</b>

1. Cyanuric Acid levels measured using standard test kit (precipitation method).

Fig. 10

## METHOD FOR REDUCING CYANURIC ACID IN RECREATIONAL WATER SYSTEMS

### RELATED APPLICATIONS

This application claims priority to and benefit of U.S. Provisional Application No. 62/073,335, filed in Oct. 31, 2014 and U.S. Provisional Application No. 62/101,741, filed on Jan. 9, 2015, the contents of which are hereby incorporated by reference in their entirety.

### INCORPORATION-BY-REFERENCE OF SEQUENCE LISTING

The contents of the text file named "BIOW-013/001US\_Sequence Listing\_ST25.txt", which was created on May 12, 2015 and is 3 KB in size, are hereby incorporated by reference in their entirety.

### FIELD OF THE INVENTION

The present invention relates to a method for treating recreational water systems with compositions comprising an oxidizable carbon species and micro-organisms, in order to reduce cyanuric acid levels in said water systems.

### BACKGROUND OF THE INVENTION

Recreational water systems such as swimming pools, spas, hot tubs, and jetted tubs, are commonly treated with chlorinated derivatives of cyanuric acid (1,3,5-triazine-2,4,6(1H, 3H,5H)-trione) in order to disinfect the water and maintain sanitary conditions. The action of these chlorinated cyanuric acid derivatives, typically referred to by the trade names di- or trichlor, is attributed to the generation of free chlorine as HOCl and OCl— arising from the hydrolytic equilibria of the various chlorinated species (O'Brien et al., 1974). When used in this way there is a gradual accumulation of residual cyanuric acid in the water (Downes et al., 1984). As the level of cyanuric acid rises, free chlorine's ability to act as a disinfectant is weakened due to increased complexation of chlorine. Above about 50 ppm cyanuric acid, the time it takes to kill bacteria in chlorinated water increases versus similarly treated water without cyanuric acid. In heated systems, such as hot tubs and spas, at even moderate levels of cyanuric acid the amount of time it takes chlorine to kill a common pathogen such as *pseudomonas aeruginosa* can be as much as one hundred times as long as similar systems without cyanuric acid.

A 2007 study by the United States Centers for Disease Control and Prevention (Shields et al., 2007) revealed that cyanuric acid significantly diminishes chlorine's ability to inactivate chlorine-resistant protozoan and cryptosporidium. Based on these findings several state and local Departments of Health have issued recommendations to the recreational water industry that cyanuric acid levels not exceed 30 ppm.

It is a common practice in the recreational water industry to reduce excess cyanuric acid levels by partially draining pools, tubs, spas, holding tanks, etc., and refilling with fresh water. This is a labor intensive and costly solution, particularly in areas affected by prolonged drought such as Southern California where the cost to replenish a typical 20,000 gallon swimming pool with fresh water is prohibitively high. Accordingly, a need exists in the recreational water industry

for compositions and methods to reduce excess cyanuric acid levels that do not require a draining and replenishing

### SUMMARY OF THE INVENTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are expressly incorporated by reference in their entirety. In cases of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples described herein are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from and encompassed by the following detailed description and claims.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing significant denitrification was observed within the first 24 hours of dosing the water with the composition of the invention.

FIG. 2 is a series of figures showing increased water clarity after dosing the water with the composition of the invention.

FIG. 3 is a graph showing decreased cyanuric acid with the composition of the invention.

FIG. 4 shows results from MS-MS analysis of in-vitro samples of cyanuric acid treated with the compositions of the invention.

FIG. 5 are growth curves showing that organisms can use cyanuric acid as a nitrogen source for growth.

FIG. 6 illustrate that Composition B from Example 1 degrades cyanuric acid.

FIG. 7 illustrates that with Composition B from Example 1 degrades cyanuric acid in water from public swimming pools

FIG. 8 illustrates that pools from southern California treated with Composition B from Example 1 have a drop in cyanuric acid concentration over time after treatment.

FIG. 9 shows the results from Florida field trials using Composition B from Example 1.

FIG. 10 shows the results comparing Compositions A, B, and C from Example 1 in actual swimming pools.

### DETAILED DESCRIPTION OF THE INVENTION

The invention provides compositions and methods for augmenting the treatment of commercial, public, and private recreational water systems such as swimming pools, spas, hot tubs, jetted tubs or the like. The composition and methods result in increased clarity of the water, decreased nitrate concentrations, decreased cyanuric acid levels, decreased biological oxygen demand (BOD), decreased total suspended solids (TSS), decreased total Kjeldahl nitrogen (TKN) and decreased fats, oils and grease (FOG) in the water. In specific embodiments, the compositions and methods are used to reduce cyanuric acid levels in recreational water systems where cyanuric acid stabilized chlorine is used as part of the routine disinfection and sanitization protocol.

In some aspects the compositions are microbial compositions. The microbial compositions are in powdered, dried form. Alternatively, the microbial compositions are in liquid form. For example, the composition includes non-pathogenic bacteria with the ability to degrade cyanuric acid.

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In certain embodiments the bacteria are derived from the genus *Bacillus*, *Lactobacillus*, *Pseudomonas*, or *Moorella*. In other embodiments the composition contain a mixture of *Bacillus* or *Lactobacillus*. In other aspects the microbial compositions contain a mixture of *Bacillus* and *Lactobacillus* bacteria. In various aspects the mixture contains at least one to seven different strains of *Bacillus*. The mixture contains at least one to four different strains of *Lactobacillus*. Optionally, the microbial compositions further include an oxidizable carbon source and/or a mixture of inorganic minerals commonly used to stimulate microbial growth.

In another aspect the composition contain containing an oxidizable carbon source and a mixture of inorganic minerals. The oxidizable carbon source is water soluble or water dispersible.

In some aspects the composition comprises at least 50%, preferably at least 75%, and most preferably at least 90% by weight of a water soluble or water dispersible oxidizable carbon source.

Preferred strains of *Bacillus* include for example, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus coagulans*, or *Paenibacillus polymyxa*. Preferred *Lactobacillus* stains includes for example, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, or *Bifidobacterium animalis*.

In a preferred embodiment the *Bacillus* is subspecies *Mojavenis*. In particularly preferred embodiments, the *Bacillus* is *Bacillus subtilis* strain 34KLB (SEQ ID NO: 1):

*Bacillus subtilis* strain 24KLB

(SEQ ID NO: 1)

AGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTAGA  
AAGGAGGTGATCCAGCCGACCTTCCGATACGGCTACCTTGTTACGACTTC  
ACCCCAATCATCTGTCCACCTTCCGGCGGTGGCTCCATAAGGTTACCTC  
ACCGACTTCGGGTGTTACAACTCTCGTGGTGTGACGGGCGGTGTGTACAA  
GGCCCGGGAACGTATTACCGCGCATGCTGATCCGCGATTACTAGCGATT  
CCAGCTTCACGCAGTCGAGTTGACAGCTGCGATCCGAAGTGAACAGATT  
TGTRGATTGGCTTAACCTCGCGTTTCGCTGCCCTTTGTTCTGTCCATTG  
TAGCAGTGTGTAGCCAGGTGATAGGGGATGATGATTGACGTCATCC  
CCACCTTCTCCGTTTGTACCGCGAGTCACCTTAGAGTGCCCAACTGAA  
TGCTGGCAACTAAGATCAAGGTTGCGCTCGTTGCGGACTTAACCAACA  
TCTCAGCAGCAGAGCTGACGACAACCATGCACACCTGTCACTCTGCCCCC  
GAAGGGGACGTCCTATCTCTAGGATTGTGAGGATGTCAAGACCTGGTAA  
GGTTCTTCGCGTTGCTTCGAATTAACACATGCTCCACGCTTGTGCGGG  
CCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCAGCGTACTCCCCAGGCGG  
AGTGCTTAATGCGTTAGCTGCAGCACTAAAGGGGCGGAAACCCCTAACAC  
TTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCG  
CTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCTT  
CGCCACTGGTGTCTCTCCACATCTCTACGCATTTACCGCTACAGTGGAA  
TTCCACTCTCCTCTCTGCACTCAAGTTCCCGAGTTTCCAATGACCTCCC  
CGGTTGAGCCGGGGCTTTACATCAGACTTAAGAAACCGCTGCGAGCCC

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-continued

TTTACGCCCAATAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGC  
TGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCC  
5 GCCCTATTGTAACGGCACTTGTCTTCCCTAACACAGAGCTTTACGATCC  
GAAAACCTTCATCACTCACGCGCGGTGTCTCCGTGAGACTTTCTGTCCATTG  
CGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT  
10 CCCAGTGTGGCGCATCACCTCTCAGGTGCGGTACGCATCGTCGCTTGGT  
GAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTG  
GTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTTCAGACAACCATCC  
15 GGTATTAGCCCCGGTTTCCCGAGTTATCCAGTCTTACAGGCAGGTTACC  
CACGTGTTACTCACCCGTCCGCGCTAACATCAGGGAGCAAGCTCCCATCT  
GTCCGCTCGACTTGATGTATTAGGCACGCCGCCAGCGTTCTGCTCTGAGCC  
ATGAACAAACTCTAAGGGCGAATTCTGAGATATCCATCACACTGGCGGCC  
20 GCTCGAGCATGCATCTAGAGGGCCCAATCGCCCTAT

In some aspects the microbial composition comprises a mixture of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus pumilus*. In another aspect the microbial composition comprises a mixture of *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum*.

In some aspects the composition comprises at least 50%, preferably at least 75%, and most preferably at least 90% by weight of a water soluble or water dispersible oxidizable carbon source. In some embodiments the composition comprises at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% by weight of a water soluble or water dispersible oxidizable carbon source.

Suitable water soluble oxidizable carbon sources include carbohydrates, proteins, polysaccharides or mixtures thereof. In preferred embodiments the water soluble carbon source comprises glucose, dextrose, fructose, erythrose, arabinose, ribose, deoxyribose, galactose, mannose, sucrose, lactose, maltose, dextrin, maltodextrin, glycerol, sorbitol, xylitol, inulin, trehalose, low molecular weight starches, modified starches, cellobiose, modified celluloses, amino acids, water soluble peptides, or mixtures thereof.

Suitable water dispersible carbon sources include emulsified fats and oils. In certain preferred embodiments the water dispersible carbon source comprises soy lecithin, emulsified vegetable oil or mixtures thereof. Other embodiments include mixtures of water soluble and water dispersible oxidizable carbon sources.

Suitable minerals include disodium hydrogen phosphate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, potassium chloride, magnesium sulfate, calcium sulfate, magnesium chloride, calcium chloride, and iron(III) chloride. The minerals comprise between 1 to 50%, 10 to 50%, 20 to 50%, 30 to 50% or 40 to 50% by weight of the composition. Preferably, the minerals comprise about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% by weight of the composition.

A preferred composition according to the invention includes about 50% by weight of a water soluble or water dispersible oxidizable carbon source and about 50% by weight of a mixture of inorganic minerals. Preferably, the water soluble or water dispersible oxidizable carbon source is dextrose and the minerals include disodium hydrogen phos-

phate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, magnesium sulfate, calcium chloride, and iron(III) chloride.

Another preferred composition according to the invention includes at least 94% by weight of a water soluble or water dispersible oxidizable carbon source and the remainder by weight of a microbial mixture. The microbial mixture is a mixture of *Bacillus*, *Lactobacillus* or both. In some aspects the microbial composition comprises a mixture of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus pumilus*. In another aspect the microbial composition comprises *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*.

A first preferred *Bacillus* mixture includes 10% by weight *Bacillus licheniformis*, 30% by weight *Bacillus pumilus*, 30% by weight *Bacillus amyloliquefaciens* and 30% by weight *Bacillus subtilis*. (referred to herein as *Bacillus Mix #1*)

A second preferred *Bacillus* mixture includes equal weights of *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens* and *Bacillus subtilis*. (referred to herein as *Bacillus Mix #2*)

A preferred *Lactobacillus* mixture includes equal weights of *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*. (referred to herein as *Lactobacillus Mix #1*)

Another preferred composition according to the invention includes at least 94% by weight of a water soluble or water dispersible oxidizable carbon source and about at least 0.1 to 1%, 0.1 to 2%, 0.1 to 3%, 0.1 to 4%, 0.1 to 5% of *Bacillus Mix# 1* and/or of *Bacillus Mix# 2*. Preferably the composition comprises about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% or 1% of *Bacillus Mix# 1* and/or of *Bacillus Mix# 2*. In some embodiments the composition also includes about at least 0.1 to 1%, 0.1 to 2%, 0.1 to 3%, 0.1 to 4%, 0.1 to 5% of *Bacillus 34KLB*. Preferably the composition comprises about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% 1%, 2%, 3%, 4% or 5% of *Bacillus 34KLB*.

In yet another preferred composition according to the invention includes at least 94% by weight of a water soluble or water dispersible oxidizable carbon source and about at least 0.1 to 1%, 0.1 to 2%, 0.1 to 3%, 0.1 to 4%, 0.1 to 5% of *Bacillus Mix# 1* and *Bacillus Mix# 2* and about at least 1 to 5% *Lactobacillus Mix #1*. Preferably the composition comprises about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% or 1% of *Bacillus Mix# 1* and *Bacillus Mix# 2* and about 1%, 2%, 3% 4% or 5% of *Lactobacillus Mix #1*.

The levels of the bacteria to be used according to the present invention will depend upon the types thereof. It is preferred that the present product contains bacteria in an amount between about  $10^5$  and  $10^{11}$  colony forming units per gram.

The microbial compositions according to the invention may be produced using any standard fermentation process known in the art. For example, solid substrate or submerged liquid fermentation under conditions which are optimized for growth of each organism. The fermented cultures can be mixed cultures or single isolates.

In some embodiments the bacteria are anaerobically fermented in the presence of carbohydrates. Suitable carbohydrates include inulin, fructo-oligosaccharide, and gluco-oligosaccharides.

When the cell density of the fermentation reaches about  $10^{11}$ – $10^{12}$  cfu/g, the individual bacteria are harvested. The

bacteria may be harvested by any known methods in the art. For example the bacteria are harvested by filtration or centrifugation.

The bacteria are dried by any method known in the art. For example the bacteria are air dried, or dried by freezing in liquid nitrogen followed by lyophilization.

The compositions according to the invention have been dried to moisture content less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, or 1%. Preferably, the composition accord the invention has been dried to moisture content less than 5%.

In some embodiments the dried powder is ground to decrease the particle size. The bacteria are ground by conical grinding at a temperature less than 10° C., 9° C., 8° C., 7° C., 6° C., 5° C., 4° C., 3° C., 1° C., 0° C., or less. Preferably the temperature is less than 4° C.

For example the particle size is less than 1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200, or 100 microns. Preferably, the freeze dried powder is ground to decrease the particle size such that the particle size is less than 800 microns. Most preferred are particle sizes less than about 400 microns. In most preferred embodiments, the dried powder has a mean particle size of 200 microns, with 60% of the mixture in the size range between 100-800 microns. In various embodiments the freeze dried powder is homogenized.

In various embodiments the microbial compositions are mixed with an inert carrier such as anhydrous dextrose, dextrose monohydrate, dendritic salt, rice bran, wheat bran, oat bran, soybean meal, rice hulls, or a mixture thereof.

The inert carrier is at a concentration of at least 60%, 70%, 75%, 80%, 85%, 90%, 95% or more. Preferably, the inert carrier is at a concentration of about between 75-95% (w/w).

In other aspects the bacterial compositions contain an organic emulsifier such as, for example, soy lecithin. The organic emulsifier is at a concentration of about 1%, 2%, 3%, 4%, 5%, 5, 7%, 8%, 9% or 10%. Preferably, the organic emulsifier is at a concentration of between 2 to 5% (w/w).

In other aspects the microbial compositions are mixed in equal proportion and added to an oxidizable carbon source. Typically, the final concentration of bacteria in the finished composition ranges from  $10^5$  to  $10^{11}$  cfu/g.

Further, if desired, the microbial compositions may be encapsulated to further increase the probability of survival; for example in a sugar matrix, fat matrix or polysaccharide matrix.

Importantly, the compositions of the invention fully disperses upon the addition to water and unlike other water treatment microbial compositions the compositions do not require a pre-activation of the bacteria, prior to use.

The compositions of the invention are used to treat both fresh and salt water commercial, municipal, industrial, and residential swimming pools, spas, hot tubs, jetted tubs and the like.

An aqueous solution of the dry composition according to the invention can be employed to increase clarity of the water, decrease nitrate concentrations, cyanuric acid levels, biological oxygen demand (BOD), total suspended solids (TSS), total Kjeldahl nitrogen (TKN) and fats, oils and grease (FOG) in body of water. The compositions of the invention may also be used to treat swimming pools and the like to remove scum and reduce algae.

Solutions of the composition can be pumped into the system to be treated or sprayed onto the surface, or into the airspace surrounding the material, or applied to a filter or other solid support through which the water to be cleaned is passed. The dry material can be mixed into a slurry or solution at the point of application and applied in a similar manner.

The invention includes methods for treating a recreational water system by contacting the water system with a composition having an oxidizable carbon source. The carbon source is water soluble or water dispersible. The method results in decreased cyanuric acid levels.

Solutions of the composition can be pumped into the system to be treated or sprayed onto the surface, or into the airspace surrounding the material, or applied to a filter or other solid support through which the water to be cleaned is passed. The dry material can be mixed into a slurry or solution at the point of application and applied in a similar manner.

The water system is dosed at a concentration range of about 0.01 to 100 ppm, preferably from 0.1 to 10 ppm.

The term "microbial, bacteria" or microbes" as used herein, refers to micro-organisms that confer a benefit. The microbes according to the invention may be viable or non-viable. The non-viable microbes are metabolically-active. By "metabolically-active" as used herein is meant that they exhibit at least some respiration or residual enzyme, or secondary metabolite activity characteristic to that type of microbe.

By the term "non-viable" as used herein is meant a population of bacteria that is not capable of replicating under any known conditions. However, it is to be understood that due to normal biological variations in a population, a small percentage of the population (i.e. 5% or less) may still be viable and thus capable of respiration and/or replication under suitable growing conditions in a population which is otherwise defined as non-viable.

By the term "viable bacteria" as used herein is meant a population of bacteria that is capable of respiring and/or replicating under suitable conditions in which respiration and/or replication is possible. A population of bacteria that does not fulfill the definition of "non-viable" (as given above) is considered to be "viable".

The term "recreational water system" as used herein is meant to include swimming pools, spas, hot tubs, jetted tubs or the like, and includes both salt water and fresh water systems.

"Treating" as used herein means inoculating water with an oxidizable carbon source and/or microbes designed to enhance efficient degradation of organic matter, cyanuric acid or both.

The compositions of the invention are manufactured by any method suitable for production of bacterial compositions. Preferably, mixtures of bacteria containing *Bacillus*, *Lactobacillus*, *Pseudomonas*, or *Moorella*., are manufactured by individually aerobically or anaerobically fermenting each organism; harvesting each organism; drying the harvested organisms, grinding the dried organisms to produce a powder combining each of the organisms into the final mixture. The *Bacillus* organisms are *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus coagulans*, or *Paenibacillus polymyxa*. The *Lactobacillus* comprises *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, or *Bifidobacterium animalis*. The mixture has a moisture content of less than about 5%; and a final bacterial concentration of between about  $10^5$ - $10^{11}$  colony forming units (CFU) per gram of the composition.

Unless stated otherwise, all percentages mentioned in this document are by weight based on the total weight of the composition.

A better understanding of the present invention may be given with the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

## EXAMPLES

## Example 1

## Preparation of the Microbial Species

The microbial species of the present invention may be made by any of the standard fermentation processes known in the art. In the following examples, both solid state and submerged liquid fermentation processes are described.

Solid State Fermentation—*Bacillus* Species

Individual purified isolates of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus coagulans*, and *Paenibacillus polymyxa* were grown-up in separate fermenters using standard aerobic submerged liquid fermentation protocols. The individual organisms were recovered from the fermenters via centrifugation, mixed together in equal proportions on a weight basis, then added to the following mixture: 1 part inulin, 2.2 parts isolated soy protein, 8 parts rice flour with 0.25% w/w sodium chloride, 0.045% w/w Calcium carbonate, 0.025% w/w Magnesium sulphate, 0.025% w/w Sodium phosphate, 0.012% w/w Ferrous sulphate and 29.6% water. This mixture was allowed to ferment for up to 5 days at 30° C. Upon completion of the fermentation, the entire mixture was freeze dried to a moisture content less than 5%, ground to an average particle size of 295 microns, with 60% of the product in the size range between 175-840 microns, and homogenized. The final microbial concentration of the powdered product is between  $10^9$  and  $10^{11}$  CFU/g.

Submerged Liquid Fermentation—*Bacillus* Species

Individual starter cultures of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus coagulans*, and *Paenibacillus polymyxa* are grown according to the following general protocol: 2 grams nutrient broth, 2 grams AmberFerm (yeast extract), and 4 grams Maltodextrin are added to a 250 ml Erlenmeyer flask. 100 milliliters distilled, deionized water was added and the flask stirred until all dry ingredients were dissolved. The flask was covered and placed for 30 min. in an Autoclave operating at 121° C. and 15 psi. After cooling, the flask was inoculated with 1 ml of one of the pure microbial strains. The flask was sealed and placed on an orbital shaker at 30° C. Cultures were allowed to grow for 3-5 days. This process was repeated for each of the micro-organisms in the mixture. This process provided starter cultures of each organism which were then used to prepare larger scale fermentations.

Individual fermenters were run under aerobic conditions at pH 7 at the temperature optimum for each species:

Microbe	Temperature Optimum
<i>Bacillus subtilis</i>	35° C.
<i>Bacillus amyloliquefaciens</i>	30° C.
<i>Bacillus licheniformis</i>	37° C.
<i>Bacillus coagulans</i>	37° C.
<i>Bacillus megaterium</i>	30° C.
<i>Bacillus pumilus</i>	32° C.
<i>Paenibacillus polymyxa</i>	30° C.

Solid State Fermentation—*Lactobacillus*

Individual purified isolates of *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Bifidobacterium animalis* were grown-up in separate fermenters using standard anaerobic submerged liquid fermentation protocols. The individual organisms were recovered from the



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fermenters via centrifugation, mixed together in equal proportions on a weight basis, then added to the following mixture: 1 part inulin, 2.2 parts isolated soy protein, 8 parts rice flour with 0.25% w/w sodium chloride, 0.045% w/w Calcium carbonate, 0.025% w/w Magnesium sulphate, 0.025% w/w Sodium phosphate, 0.012% w/w Ferrous sulphate and 29.6% water. This mixture was allowed to ferment for up to 5 days at 30° C. Upon completion of the fermentation, the entire mixture was freeze dried to a moisture content less than 5%, ground to an average particle size of 295 microns, with 60% of the product in the size range between 175-840 microns, and homogenized. The final microbial concentration of the powdered product is between 109 and 1011 CFU/g.

#### Submerged Liquid Fermentation—*Lactobacillus*

Individual, purified isolates of *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Bifidobacterium animalis* were grown-up in separate fermenters using standard anaerobic submerged liquid fermentation protocols. After fermentation the individual cultures were filtered, centrifuged, freeze dried to a moisture level less than about 5%, then ground to a mean particle size of 295 microns, with 60% of the product in a size range between 175-840 microns. The individual dried microbial cultures were then mixed in equal proportion by weight to obtain the microbial composition of the present invention. The final microbial concentration of the mixed powdered product is between 10<sup>9</sup> and 10<sup>11</sup> CFU/g.

#### Example 2

##### Formulation of Swimming Pool Treatment Products

The following formulations were prepared by dry blending the ingredients in a ribbon blender (all percentages are by weight):

COMPOSITIONS								
Ingredients	A	B	C	D	E	F	G	H
Microbial Composition from Example 1	5	5	10	10	25	25		
<i>Bacillus subtilis</i> 34KLB produced via submerged liquid fermentation according to Example 1 B							1	10
Dextrose	95		90		75		99	90
Nutri-Sure™		95		90		75		

#### Example 3

##### Denitrification of Swimming Pool Water

The composition of Example 2H was used to reduce the nitrate level in a swimming pool. 100 grams of the composition of Example 2H were added to the skimmer of a 15,000 gallon swimming pool with initial nitrate level of about 25 ppm. Significant denitrification was observed within the first 24 hours of dosing. FIG. 1.

#### Example 4

##### Water Clarification

The conditions of a typical swimming pool (chlorine level, temperature) were mimicked in the laboratory using 10 gal-

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lon aquaria fitted with a filter and circulation pump. The composition of Example 2F was evaluated for its ability to clarify the water after addition of 250 ppm Sunscreen. Results are in FIG. 2.

Significant clarification is noted in as little as 24 hours. Separate respirometer analysis with sunscreen as a substrate showed significant CO<sub>2</sub> generation and O<sub>2</sub> consumption when the composition of Example 2F is added.

#### Example 5

##### Biodegradation of Cyanuric Acid

The composition of Example 2E was evaluated for its ability to degrade cyanuric acid. A solution of 1 gram of the composition of Example 2E in 1 L of Deionized Water was prepared and allowed to stand for 40 hours at room temperature. 5 mls of this solution were then added to 500 mls of water containing minimal media and 50 ppm cyanuric acid. The degradation of cyanuric acid was followed by HPLC. Results are shown in FIG. 3

#### Example 6

##### Cyanuric Acid Control in Swimming Pools

The composition of Example 2E was evaluated in swimming pool applications. Testing was conducted in 6 fresh water swimming pools and 3 salt water pools in Southern California. 100 grams of the composition of Example 2E were added to each pool and the cyanuric acid level determined as a function of time using a commercially available cyanuric acid test kit. Results are summarized in Table 1:

TABLE 1

Cyanuric Acid Control in Swimming Pools			
	Cyanuric Acid at T = 0	Cyanuric Acid at 1 week	% Reduction
Fresh Water Pool			
Pool #1	100 ppm	57.5 ppm	42.5
Pool #2	100 ppm	65 ppm	35
Pool #3	50 ppm	20 ppm	60
Pool #4	100 ppm	67.5 ppm	32.5
Pool #5	>125 ppm	100 ppm	>20
Pool #6	100 ppm	65 ppm	35
Salt Water Pool			
Pool #1	>100 ppm	40 ppm	>60
Pool #2	>200 ppm	60 ppm	>70
Pool #3	>500 ppm	120 ppm	>75

#### Example 7

##### Preparation of Cyanuric Acid Reducing Compositions

The following compositions were prepared (all percentages are by weight):

	Composition A	Composition B	Composition C	Composition D	Composition E	Composition F
Dextrose	100%	94.30%	95.0%	97.0%		50%
Maltodex					94.0%	
Bacillus Mix #	0.43%		1.0%	0.44%		
Bacillus Mix #	0.43%		1.0%	0.44%		
Lactobacillus Mix #1		4.40%			4.40%	
B. Subtilis 34KLB		0.44%	5.0%	1.0%	0.72%	
Mineral Mix						50%

Where *Bacillus* Mix #1 comprises 10% by weight *Bacillus licheniformis*, 30% by weight *Bacillus pumilus*, 30% by weight *Bacillus amyloliquefaciens*, and 30% by weight *Bacillus subtilis* subspecies *Mojavensis* and has an activity  $\geq 10^{11}$  cfu/g; *Bacillus* Mix #2 comprises equal weights of *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, and *Bacillus subtilis* with an activity  $\geq 10^9$  cfu/g; and, *Lactobacillus* Mix #1 comprises equal weights of *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum* at a concentration  $\geq 10^{10}$  cfu/g. The *Bacillus subtilis* 34KLB had an activity  $\geq 10^{10}$  cfu/g.

The mineral mix comprises the following:

Mineral	Wt. %
KH <sub>2</sub> PO <sub>4</sub>	12%
K <sub>2</sub> HPO <sub>4</sub>	31%
Na <sub>2</sub> HPO <sub>4</sub> *2H <sub>2</sub> O	48%
CaCl <sub>2</sub> *2H <sub>2</sub> O	5%
MgSO <sub>4</sub> *7H <sub>2</sub> O	3%
FeCl <sub>3</sub> *6H <sub>2</sub> O	1%

### Example 8

#### In Vitro Cyanuric Acid Reduction

Compositions A and B from Example 7 were tested for their ability to degrade cyanuric acid in vitro. Stock solutions of Cyanuric acid were prepared by dissolving 100 mg cyanuric acid in 1 liter of hot autoclaved DI water. Compositions A and B were dosed at 1 gram/L into separate 100 mls aliquots of the stock cyanuric acid solutions then incubated in an incubating shaker at 30° C./150 rpm. 10 ml aliquots were pulled from each sample at times 0, 3, 6, 8 and 24 hours then frozen to shut down microbial activity. The frozen samples were analyzed by MS-MS. Results are shown in FIG. 4:

POOL	TYPE	TEMPERATURE	CHLORINE	ALKALINITY	PH	CYA	FILLER	GALLONS
1	Salt water Vinyl liner	START: 55° 24 HR: 58°	START: 2.0 24 HR: 1.0	START: 100 24 HR: 90	START: 7.4 24 HR: 7.4	START: 70 3 HR: N/C 24 HR: 50	DE	20,000
2	Fresh water Vinyl liner	START: 55° 24 HR: 59°	START: 5 24 HR: 3.0	START: 120 24 HR: 120	START: 7.2 24 HR: 7.4	START: 140-150 3 HR: 100-110 24 HR: 80	SAND	14,000
3	Salt water Vinyl liner	START: 58° 24 HR: 53° 48 HR: 55°	START: 3.0 24 HR: 3.0 48 HR: 3.0	START: 90 24 HR: 90 48 HR: 90	START: 7.2 24 HR: 7.4 48 HR: 7.4	START: 75-80 24 HR: 45-50 DAY 5: 35-40		20,000
6	Fiber Glass	START: 65° 3 HR: 85° 26 HR: 85° 65 HR: 65°	START: 3.0 3 HR: 4.0 72 HR: 4	START: 100 3 HR: 120 72 HR: 100	START: 7.6 3 HR: 7.8 72 HR: 7.8	START: 200+ 3 HR: 100 22 HR: 80-90 26 HR: 70 72 HR: 50-70	SAND	6,500
7	Fiber Glass	START: 60° 3 HR: 62° 24 HR: 58°	START: 2.0 3 HR: 2.0 24 HR: 2.0	START: 80 2 HR: 80 24 HR: 80	START: 7.4 3 HR: 7.2 24 HR: 7.4	START: 200+ 3 HR: N/C 24 HR: 90 48 HR: 70	SAND	5,000

As expected, in sterile systems, addition of an oxidizable carbon source alone (Composition A) is not sufficient to cause reduction of cyanuric acid. However, in combination with select microbial species (Composition B), 65% of the initial cyanuric acid is removed after 24 hours incubation.

### Example 3

#### In-vivo Cyanuric Acid Reduction

Compositions A, B, and C from Example 7 were tested for their ability to reduce cyanuric acid in recreational swimming pools. For this analysis cyanuric acid levels were measured using a test kit common to the pool industry. The kit comprises a plastic tube into which a sample of pool water is added. A reagent solution comprising a low level of melamine is added to the pool water and the sample agitated for 1-2 minutes. Cyanuric acid levels are recorded visually using a dipstick calibrated between 20-100 ppm. 8 Ounces of the compositions from Example 7 were added into the skimmers of three separate pools. Results are shown below:

Pool #1	Pool #2	Pool #3
4.3 ppm Composition A Pool Capacity = 14,000 gal. 82° F. pH: 7.8 Initial CYA level = +150 ppm CYA level at 25 hours = 55 ppm % CYA Reduction >63%	3.7 ppm Composition B Pool Capacity = 16,000 gal. 80° F. pH: 7.6 Initial CYA level = 120 ppm CYA level at 25 hours = 65 ppm % CYA Reduction >45%	3.7 ppm Composition C Pool Capacity = 16,000 gal. 85° F. pH: 7.6 Initial CYA level = 120 ppm CYA level at 25 hours = 75 ppm % CYA Reduction >37%

The Compositions were further tested for their ability to reduce cyanuric acid in recreational swimming pools. Results are shown below:

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POOL	TYPE	TEMPERATURE	CHLORINE	ALKALINITY	PH	CYA	FILLER	GALLONS
8	Cement	START: 58° 24 HR: 58°	START: 5 24 HR: 4 48 HR: 10	START: 120 24 HR: 120 48 HR: 100	START: 7.4 24 HR: 7.4 48 HR: 7.4	START: 300+ 24 HR: 100-110 48 HR: 80-90	SAND	12,000
9	Commercial Gunnite	START: 60° 21 HR: 62° 72 HR: 60°	START: 1.0 21 HR: 2.0 72 HR: 2	START: 120 21 HR: 120 72 HR: 120	START: 7.8 21 HR: 7.8 72 HR: 7	START: 180 21 HR: 100 72 HR: 60	SAND	108,000

## OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is

intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

## SEQUENCE LISTING

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<211> LENGTH: 1668

<212> TYPE: DNA

<213> ORGANISM: *Bacillus subtilis*

<400> SEQUENCE: 1

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tgggtgtgacg ggcggtgtgt acaaggcccg ggaacgtatt caccgcgga tgctgatccg      240
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We claim:

1. A composition comprising
  - a. between 75-95% w/w of anhydrous dextrose or dextrose monohydrate;
  - b. a mixture of *Bacillus* bacterial species comprising *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus* having a bacterial concentration of at least  $1 \times 10^6$  colony forming units (CFU) per gram of the mixture, wherein each of the *Bacillus* species are individually fermented aerobically, dried and ground to an average particle size of about 200 microns, and wherein the *Bacillus subtilis* comprises *Mojavensis*;
  - c. a mixture of *Lactobacillus* bacterial species comprising *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum* having a bacterial concentration of at least  $1 \times 10^6$  colony forming units (CFU) per gram of the mixture, wherein each of the *Lactobacillus* species are fermented anaerobically, dried, and ground to an average particle size of about 200 microns.
2. The composition of claim 1, wherein the bacterial species are non-pathogenic.
3. The composition of claim 1, wherein the *Bacillus subtilis* includes *Bacillus subtilis* 34 KLB.
4. The composition of claim 1, wherein each of the *Lactobacillus* bacterial species are present in equal amounts by weight.
5. The composition according to claim 1, wherein the composition further comprises an inorganic mineral that stimulates bacterial respiration and growth.
6. The composition according to claim 5, wherein the inorganic mineral is disodium hydrogen phosphate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, potassium chloride, magnesium sulfate, calcium sulfate, magnesium chloride, calcium chloride, or iron(III) chloride.
7. A method for reducing cyanuric acid concentration on recreational water systems comprising contacting a pool's filtration system with the composition of claim 1.
8. A composition comprising
  - a. between 75-95% w/w of anhydrous dextrose or dextrose monohydrate;
  - b. at least 1% w/w of a mixture containing *Bacillus* bacterial species comprising *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus*, wherein each of the *Bacillus* species are individually fermented aerobically, dried and ground to an average particle size of about 200 microns, and wherein the *Bacillus subtilis* comprises *Mojavensis*;
  - c. at least 4% w/w of a mixture containing *Lactobacillus* bacterial species comprising *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum*, wherein each of the *Lactobacillus* species are fermented anaerobically, dried, and ground to an average particle size of about 200 microns.
9. The composition of claim 8, wherein a bacterial concentration of the *Bacillus* mixture is at least  $1 \times 10^6$  colony forming units (CFU) per gram of the mixture and the bacterial concentration the *Lactobacillus* mixture is at least  $1 \times 10^6$  colony forming units (CFU) per gram of the mixture.
10. The composition of claim 9, wherein at least 15% of the *Bacillus* CFUs are *Bacillus subtilis* 34 KLB.
11. A composition comprising:
  - a. at least 0.4% w/w/ of a first *Bacillus* mixture comprising *Bacillus subtilis* subsp *mojavensis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus*;
  - b. at least 0.4% w/w/ of a second *Bacillus* mixture comprising *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus*;
  - c. at least 0.4% w/w/ of a mixture of *Bacillus subtilis* 34 KLB; and
  - d. at least 4% w/w of a *Lactobacillus* mixture comprising *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus plantarum*.
12. The composition of claim 11, wherein the remainder of the composition comprise an oxidizable carbon source.
13. The composition of claim 12, wherein the oxidizable carbon source is anhydrous dextrose or dextrose monohydrate.
14. The composition of claim 11, wherein the *Lactobacillus* in the *Lactobacillus* mixture are present in equal proportions by weight.
15. The composition of claim 11, wherein the *Bacillus subtilis* subsp *mojavensis*, the *Bacillus amyloliquefaciens*, and the *Bacillus pumilus* in the first *Bacillus* mixture are present in equal proportions by weight.

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